Investigating the function of MHC Class I in *Xenopus laevis* tadpoles

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ABSTRACT

Xenopus laevis, is an excellent model organism for transgenesis and immunological research due to their large eggs and the similarities between mammalian and amphibian immune systems. MHC Class I is found on nearly all cells and educates T cells to recognize between self and non-self which is an essential immune system function. However, tadpoles don't have detectable MHC Class I expression while adult frogs do. Despite this tadpoles are still immunocompetent; thus the role of MHC Class I in tadpoles is unknown. To investigate MHC Class I function we inactivated the corresponding gene in *Xenopus laevis* using CRISPR/Cas9. The CRISPR/Cas9 gene editing system has two components; a guide RNA that targets a specific DNA sequence and the Cas9 protein that cuts the DNA. After the DNA is cut, the cell repairs the DNA break, which introduces mutations that theoretically inactivate this gene. Previously, we generated transgenic tadpoles with potentially inactivated MHC Class I genes, and we are in the process of determining the success of our transgenesis. To detect MHC Class I inactivation we will use PCR to amplify the MHC class I gene from genomic DNA of the transgenic tadpoles which will then be sent for sequencing.

INTRODUCTION

Xenopus laevis were used as the model organism (Figure 2B) because the immune system of this species is analogous to a human's, research into their immune system can provide insights for the human immune system.

Major Histocompatibility Complex I (MHC class I) is a protein made up of three different domains α_1, α_2, α_3, that associate with $\boldsymbol{\beta}$2-microglobulin in order to form a stable and functional protein at the cell surface (Figure 1A). Two important domains of this protein are α_1 and α_2 which make up the antigen binding site therefore these two domains are quite variable (Figure 1B). MHC class I is found on the surface of all nucleated cells in the human body and as a result, MHC class I is a protein that allows the immune system to distinguish self and nonself. This mechanism contributes to self-tolerance, which allows the body to produce immune cells that do not attack itself. Another important function of this protein is the activation of other immune cells that respond to infection of the body from pathogens. Therefore, loss of MHC class I would disrupt the immunocompetency of the organism as it cannot fully protect itself from certain foreign invaders and it may also lead to autoimmunity.

In *Xenopus* MHC Class I functions similar to the human MHC class I. However the expression of this protein is different during the different stages of development. Unlike in the adult stage, MHC class I protein and mRNA are undetectable in *Xenopus* tadpoles. Thus, it can be implied that through loss of MHC class I tadpoles undergo systemic autoimmunity and die without reaching their adult stage. However, this is not the case as tadpoles continue to develop into adult frogs. While tadpoles lack such an essential protein they are still immunocompetent. Therefore the function of MHC class I in tadpoles is unknown. In order to determine the role of MHC class I in tadpoles, CRISPR/Cas9 technology was utilized to mutate the corresponding gene in tadpoles (Figure 3A). CRISPR/Cas9 was originally found in bacteria as a method of protection against bacteriophages, but has been adapted for genome editing. It is used to inactivate the gene of interest by introducing a cut at the corresponding gene using a small guide RNA (sgRNA) (Figure 3A). When CRISPR/Cas9 makes a cut it can either make a single-stranded cut (Figure 3B) or a double-stranded cut (Figure 3C). A single-stranded cut can regain its original composition given the nucleotide sequence on the remaining strand, while a double-stranded cut does not have any information to fix itself. In order to fix a double stranded break, cells use a DNA repair method called non-homologous end joining (NHEJ) where there is insertion and deletions of random nucleotides causing a mutation, therefore inactivating the gene.

MATERIALS AND METHODS

sgRNA Design: Three requirements were selected for in the sgRNA using the design tool CRISPR direct (https://crispr.dbcls.jp/). These were the presence of a PAM sequence, a 50% GC content, and an annealing temperature of around 70 °C. The sequences had to be checked for nonspecific matches and therefore run through the BLAST tool by the NCBI. Only the sgRNAs that closely matched the *Xenopus laevis* sequence for α_1 and α_2 were used.

sgRNA Preparation: The ordered sgRNA sequence was flanked by a 5' T7 promoter and a 3' universal primer with an overhang. We used annealing PCR where the 3' overhand with the universal primer was able to bind to a double stranded DNA copy and act as a reverse primer for amplification of the sgRNA. The sgRNA was checked by PCR, and the Nanodrop spectrophotometer was used to quantify the concentration and purity.

Transgenesis: Transgenesis was done at the University of Rochester. Embryos were first de-jellied in HCL-cysteine in 0.1X MBS. Then, they were washed with 0.1X MBS three times, and placed in 0.3X MBS with Ficoll. Using a pressurized needle, each embryo was injected with 10 nL mixture containing 8 ng of Cas9 protein and 200 pg/nL of either sgRNA into the germinal vescile of the animal pole that develops from the vegetal pole. This solution was prepared just before injections. The embryos were allowed to hatch in a solution of 0.3X MBS with 5 µg/mL gentamicin at 18°C for 3 days. Media was changed daily and dead embryos were removed. Survival rate was determined upon hatching (day 3) and surviving tadpoles were left to develop normally. Tadpoles that died were collected for DNA extraction and MHC class I knockdown analysis. Control animals were ones that were only dejellied but not injected.

Primer Design: Certain requirements had to be met for the primer designs. The GC content should be about 50% and abstain from long strings of C and G nucleotides, as well as similar annealing temperatures, and no hairpin structures. Primer 3 design tool (https://bioinfo .ut.ee/primer3/) was used to determine both the forward and reverse primers to avoid nonspecific binding; they were also run through BLAST tool by NCBI.

Table 1. Survival rates of injected eggs and de-jellied controls for transgenic experiments using different sgRNAs

Target: MHC Class I, α_1 domain			
Group	Starting Population	3 Days Post-Injection	Survival Rate (%)
Injected	149	39	26.2
De-jellied Control	1189	217	18.3

Target: MHC Class I, α_1 domain			
Group	Starting Population	3 Days Post-Injection	Survival Rate (%)
Injected	34	0	0
De-jellied Control	300	0	0

Target: MHC Class I, α_1 and α_2 domain			
Group	Starting Population	3 Days Post-Injection	Survival Rate (%)
Injected	108	13	12
De-jellied Control	361	119	33

Target: MHC Class I, α_1 and α_2 domain			
Group	Starting Population	3 Days Post-Injection	Survival Rate (%)
Injected	143	8	5.6
De-jellied Control	84	5	5.9

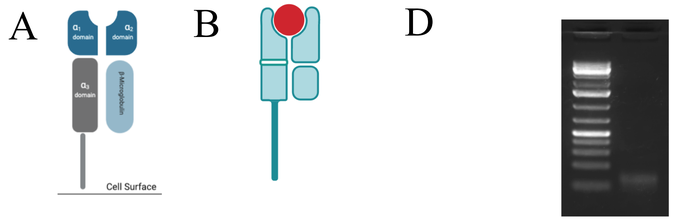

Figure 1. MHC class I protein and MHC class I sgRNA Detection by Gel Electrophoresis. (A) MHC class I protein showing the different domains and conserved regions. Image created using BioRender. (B) MHC class I domains α_1 and α_2 and their interaction with antigen. Image created using BioRender. (C) The sequence for the 3' designed MHC class I sgRNA for both the α_1 and α_2 is shown as well as the target sequence in the DNA for each region. Design tool used CRISPRdirect. (D) Top: The α_1 domain sgRNA run on an agarose gel and subject to electrophoresis. The lanes left to right show the DNA markers and the sgRNA for α_1 domain. Bottom: The α_2 domain sgRNA run on an agarose gel electrophoresis. The lanes, left to right, contain the DNA marker and two α_2 domain sgRNA samples.

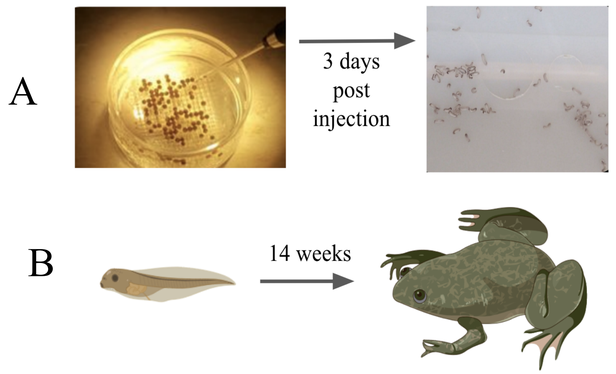

Figure 2. Transgenesis Experiment Procedure. (A) Newly fertilized eggs are dejellied, placed in ficoll media, and placed on an injection plate lined with mesh to stabilize them while injecting. The 10 nL CRISPR solution containing 8 ng of Cas9 protein and 200 pg/nL of either sgRNA is injected into the germinal vesicle of the dejellied single cell fertilized eggs. The embryos are then allowed to hatch; their progress is pictured three days post injection. (B) The development of a tadpole generally takes 14 weeks to develop into a mature adult frog. Images created by BioRender.

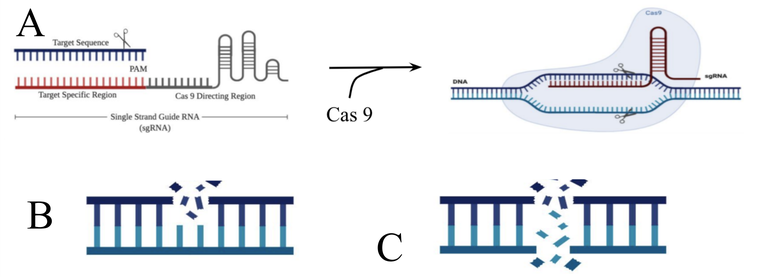

Figure 3. CRISPR/Cas9 Genome Editing. (A) The CRISPR/Cas9 complex is made up of the Cas9 protein and takes in a sgRNA template. It uses the sgRNA to cut the specific gene sequence target. (B) The DNA cut can be single-stranded, and easily repaired by the cell by utilizing base pairing of the remaining strand as a template. (C) The DNA cut can also be double-stranded, and this way the cell has no previous information for which to fix the DNA, so it can utilize NHEJ and insert/delete random nucleotides.

CONCLUSIONS

- No significant difference in survival between injected and de-jellied tadpoles 3 days post injection.
- No significance in toxicity from the sgRNAs given the data from the control and experimental tadpoles.

FUTURE RESEARCH

- Take tissue samples of surviving animals from previous transgenic experiments along with the samples we already have from the non surviving embryos to see if they have mutations in the α_1 or α_2 region of MHC class I by extracting DNA and PCR analysis followed by an ExoSAP-IT purification kit which will allows us to directly sequence our PCR products using DNA.
- Sequence analysis to see if we have knockdown of MHC class I.
- Only about 1 in 5 small sgRNAs are successful, therefore we have already generated more sgRNAs that will be tested.
- Transgenesis experiments with different sgRNAs to determine their success rate. Surviving embryos will be collected 24 hours post injection, DNA will be extracted and sequenced for the mutation on the corresponding region of the MHC class I gene.

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6. Transgenesis Protocol from the lab of Dr. Robert at the University of Rochester

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